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Aerobic Degradation of 2,4,6-Trinitrotoluene by *Enterobacter cloacae* PB2 and by Pentaerythritol Tetranitrate Reductase

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***Enterobacter cloacae* PB2 was originally isolated on the basis of its ability to utilize nitrate esters, such as pentaerythritol tetranitrate (PETN) and glycerol trinitrate, as the sole nitrogen source for growth. The enzyme responsible is an NADPH-dependent reductase designated PETN reductase. *E. cloacae* PB2 was found to be capable of slow aerobic growth with 2,4,6-trinitrotoluene (TNT) as the sole nitrogen source. Dinitrotoluenes were not produced and could not be used as nitrogen sources. Purified PETN reductase was found to reduce TNT to its hydride-Meisenheimer complex, which was further reduced to the dihydride-Meisenheimer complex. Purified PETN reductase and recombinant *Escherichia coli* expressing PETN reductase were able to liberate nitrogen as nitrite from TNT. The ability to remove nitrogen from TNT suggests that PB2 or recombinant organisms expressing PETN reductase may be useful for bioremediation of TNT-contaminated soil and water.**

A large number of sites worldwide are heavily contaminated with explosives, particularly 2,4,6-trinitrotoluene (TNT), due to the manufacture and/or testing of munitions. Bioremediation may offer an attractive means of decontaminating such sites; unfortunately, TNT is notoriously recalcitrant to complete biodegradation (10). TNT can, however, be reduced by bacteria under anaerobic conditions, yielding hydroxylamino and amino derivatives, some of which also prove toxic (13). The enzymes responsible for the reduction of the nitro groups of TNT are designated nitroreductases, and the genes have been cloned from several bacteria, including *Enterobacter cloacae* (3). Duque et al. (4) reported the isolation and characterization of *Pseudomonas* sp. strain A, capable of aerobic growth with TNT as the sole nitrogen source. It was initially reported that TNT was denitrated to produce dinitrotoluenes, mononitrotoluenes, and eventually toluene (4, 6); however, a more recent report suggests that this is not the case and that sustained growth of this organism with TNT as the sole nitrogen source may not occur (15). TNT was also reduced unproductively to give aminodinitrotoluenes, diaminonitrotoluenes, and tetranitroazoxytoluenes. Similarly, Vorbeck et al. (15) isolated from TNT-contaminated soil two organisms which initially appeared capable of utilizing TNT as the sole nitrogen source; however, growth at the expense of TNT diminished with serial subculturing, leading to doubt as to whether these strains were genuinely capable of liberating nitrogen from TNT.

E. cloacae PB2 was isolated on the basis of its ability to grow with nitrate ester explosives, such as pentaerythritol tetranitrate (PETN) and glycerol trinitrate, as the sole nitrogen source (2). The enzyme responsible for this ability was found to be an NADPH-dependent reductase, designated PETN reductase, which reductively liberates nitrite from PETN and glycerol trinitrate. The structural gene encoding PETN reductase,

designated *onr* for organic nitrate reductase, has been cloned and overexpressed in *Escherichia coli* (5).

Here we report that *E. cloacae* PB2 is also capable of growth with TNT as the sole source of nitrogen and that purified PETN reductase is capable of reducing the aromatic ring of TNT and causing the liberation of nitrite. To the best of our knowledge, this is the first report of the reduction of the aromatic ring of TNT or of the liberation of nitrogen from TNT by a purified enzyme.

MATERIALS AND METHODS

Reagents. PETN and TNT were provided by the Defence Evaluation and Research Agency (Fort Halstead, Sevenoaks, United Kingdom). Other materials were obtained from Sigma Chemical Co. (Poole, Dorset, United Kingdom) or other suppliers and were of analytical or higher grade.

Organisms and growth conditions. *E. cloacae* PB2 was originally isolated from explosive-contaminated soil on the basis of its ability to grow with nitrate esters as the sole nitrogen source (2). *E. cloacae* PB2 was grown in a minimal medium with the following composition: 19.5 mM KH₂PO₄, 30.5 mM Na₂HPO₄, and 4 ml of trace elements (stock concentrations of 0.5 M HCl, 25 mM MgO, 20 mM CaCO₃, 20 mM FeSO₄, 5 mM ZnSO₄, 5 mM MnSO₄, 1 mM CuSO₄, 1 mM CoSO₄, and 1 mM H₃BO₃)/liter. The carbon source was 22 mM D-glucose. Incubation was at 30°C with rotary shaking at 160 rpm.

The construction of plasmid pONR1, which directs high expression of PETN reductase, has been described elsewhere (5). *E. coli* JM109 and *E. coli* BLR (DE3) were grown in SOB medium (12) at 37°C with rotary shaking at 170 rpm.

Cell growth, as judged by protein concentration, in *E. cloacae* PB2 minimal medium cultures was estimated as follows. To culture samples, 0.05 volume of 10 M KOH was added to lyse cells. After 15 min of incubation at room temperature the sample was neutralized by the addition of 0.043 volume of concentrated HCl (36.5% [wt/vol] in water), and the concentration of soluble protein was estimated with the Coomassie blue-based reagent provided by Pierce (Rockford, Ill.). Bovine serum albumin was used as a standard.

Cloning and overexpression of *E. cloacae* nitroreductase. The nitroreductase gene from *E. cloacae* NCIMB10101, the type strain of the species, was cloned by PCR based on the published sequence data of Bryant et al. (3). *E. cloacae* NCIMB10101 was obtained from the National Collection of Industrial and Marine Bacteria, Aberdeen, United Kingdom. Genomic DNA was prepared as described by Ausubel et al. (1). The primers used were as follows (the bases corresponding to start and stop codons are underlined): forward, A-GGA-GTT-CAT-ATG-GAT-ATC-ATT-TCT-GTC; reverse, G-CTC-TAG-AAT-TCA-GCA-CTC-GGT-CAC-AAT.

In the forward primer, bases 11 to 28 correspond to the first 18 bases of the nitroreductase gene, bases 1 to 5 constitute a ribosome-binding site, and bases 8 to 13 introduce an *Nde*I restriction site at the start codon. In the reverse primer, bases 28 to 11 are complementary to the last 18 bases of the gene, bases 7 to 12 introduce an *Eco*RI restriction site, and bases 2 to 7 introduce an *Xba*I restriction site.

PCR was performed with BioTaq polymerase (Bioline). The annealing tem-

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perature was 50°C. The product was digested with *Nde*I and *Eco*RI and ligated into pT7-7 (U.S. Biochemical Corporation, Cleveland, Ohio) cut with the same enzymes. This vector bears a T7 RNA polymerase-dependent promoter and ribosome-binding site adjacent to the *Nde*I site. The resulting construct was designated pNITRED1.

Enzyme preparation. PETN reductase was prepared from *E. coli* JM109/pONR1 and purified as described previously (5). Nitroreductase was prepared from *E. coli* BLR(DE3)/pNITRED1. Cells were grown in SOB medium (12) to an optical density between 0.5 and 1.0 at 600 nm, and protein production was then induced by the addition of isopropyl- β -D-thiogalactoside to a final concentration of 0.4 mM. After a further 3 to 4 h of incubation at 37°C, the cells were harvested and cell extracts were prepared as described previously for PETN reductase (5). Nitroreductase represented approximately 10% of soluble protein in extracts, as judged by specific activity (approximately 40 U/mg) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Nitroreductase was not further purified.

Enzyme activity assays. PETN reductase activity was assayed in 50 mM phosphate buffer, pH 7.0, with 0.2 mM NADPH and 0.05 mM PETN (added from a stock solution of 50 mM PETN in acetone) at 30°C, except where otherwise specified. One unit of activity was defined as that amount of activity oxidizing 1 μ mol of NADPH per min under these conditions. Nitroreductase activity was assayed in 50 mM phosphate buffer, pH 7.0, with 0.2 mM NADPH and 0.1 mM TNT (added from a stock solution of 50 mM TNT in acetone) at 30°C. One unit of activity was defined as that amount of activity oxidizing 1 μ mol of NADPH per min under these conditions.

Nitrite production was assayed colorimetrically with a modification of Griess reagent as follows. To a 600- μ l sample containing 0 to 100 μ M nitrite, 1.5 μ l of 10 mM phenazine methosulfate was added to catalyze oxidation of NADPH, which would otherwise interfere with color development. The sample was allowed to stand for 10 min at room temperature, and then 200 μ l of 10 mg of sulfanilamide/ml in 0.68 M HCl and 40 μ l of 10 mg of *N*-(1-naphthyl)ethylenediamine/ml in water were added. After being mixed, the sample was allowed to stand for a further 10 min to ensure stable color formation. Absorbance at 540 nm was measured. Sodium nitrite was used as a standard.

Chromatography. TNT and metabolites were detected and quantified by high-performance liquid chromatography (HPLC) analysis with a Waters HPLC system (model 510 pump, model 712 sample processor, and model 994 photodiode array detector) fitted with a Techsphere 50DS reverse-phase column (HPLC Technology, Macclesfield, United Kingdom). The mobile phase consisted of 60% (vol/vol) methanol and 40% (vol/vol) water and was delivered at a flow rate of 1.0 ml/min. Compounds were detected at 260 nm. This solvent system resolved TNT, 2,6-dinitrotoluene, 2,4-dinitrotoluene, 2-nitrotoluene, and 4-nitrotoluene (retention times ranged from 8.6 to 14.3 min). For ion-pair HPLC, the same column was used, with a mobile phase consisting of 45% (vol/vol) acetonitrile and 55% (vol/vol) 20 mM tetrabutylammonium phosphate buffer, pH 7. Peaks were detected at 260 and 500 nm, and UV-visible spectra of peaks were measured with a Waters 994 programmable photodiode array detector.

Gas chromatography was performed with a Perkin-Elmer 8410 gas chromatograph equipped with a 30-m by 0.25-mm 1-mm DB-1 column (J&W Scientific, Folsom, Calif.). The carrier gas was nitrogen, and the temperature was raised from 100°C to 230°C at 5°C/min. This system resolved TNT, 2,6-dinitrotoluene, 2,4-dinitrotoluene, 2-nitrotoluene, and 4-nitrotoluene (retention times ranged from 9.1 to 22.4 min).

RESULTS

Growth of *E. cloacae* PB2 with TNT as the sole nitrogen source. Growth of *E. cloacae* PB2 with TNT as the sole nitrogen source was assessed in a mineral salt medium with glucose as the carbon source. As an inoculum, *E. cloacae* PB2 was grown for 2 days at 30°C with 5 mM NaNO₂ as the nitrogen source. To 50 ml of medium containing no nitrogen and with 0.5 or 1.0 mM TNT or 1, 2, or 3 mM NaNO₂ as the nitrogen source, 0.5 ml of inoculum was added. The growth curves obtained are shown in Fig. 1. Growth, estimated by turbidity and protein concentration, was observed in the presence of TNT or NaNO₂ and was proportional to the amount of the nitrogen source present in the growth medium. Viable cell counts, however, indicated considerably lower growth with TNT than with nitrite. After 15 days of growth, viable cell counts were as follows: no nitrogen, 16×10^6 CFU/ml; 0.5 mM TNT, 22×10^6 CFU/ml; 1.0 mM TNT, 31×10^6 CFU/ml; 1.0 mM nitrite, 63×10^6 CFU/ml; 2.0 mM nitrite, 420×10^6 CFU/ml; 3.0 mM nitrite, 920×10^6 CFU/ml. The relatively small increase in cell numbers, compared to the proportionally greater increases in turbidity and protein, may be due to ag-

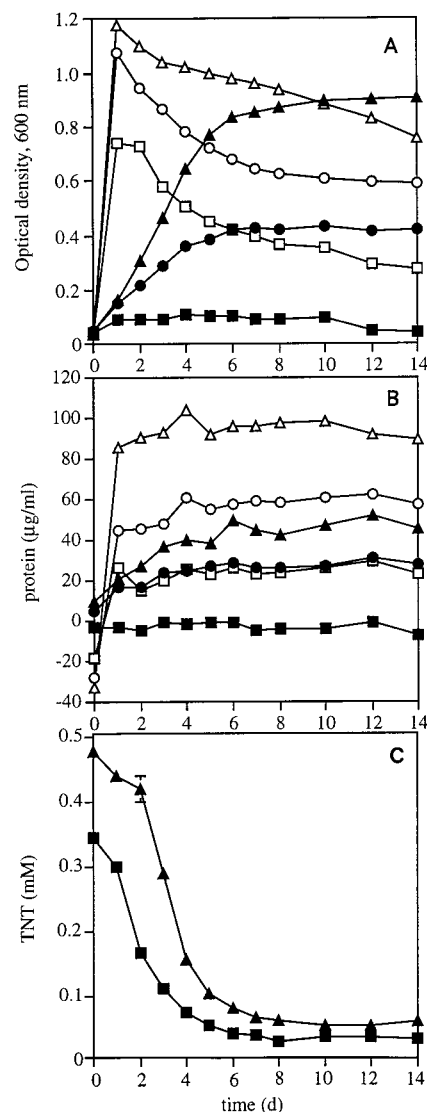


FIG. 1. Growth of *E. cloacae* PB2 with TNT as sole nitrogen source. (A) Optical density at 600 nm. (B) Protein concentration. Note that the presence of nitrite in certain cultures at time zero led to anomalously low results. It is assumed that by $t = 1$ day the nitrite concentration had fallen to a sufficiently low level that results are reliable. The results shown are the means of two or three independent measurements; standard errors were generally less than 1%, and none exceeded 2.1% of the mean. (C) TNT concentration. The results shown are the means of two independent measurements. Standard errors were generally less than 5% and in only one case exceeded 10% of the mean. The error bars indicate one standard error. Solid squares, no nitrogen source; solid circles, 0.5 mM TNT; solid triangles, 1.0 mM TNT; open squares, 1 mM NaNO₂; open circles, 2 mM NaNO₂; open triangles, 3 mM NaNO₂.

glomeration of cells or reduced cell division due to the toxicity of TNT or its metabolites. Serial subcultures with TNT as the sole nitrogen source displayed comparable turbidity increases for at least two transfers, although protein levels and cell numbers were not measured. In similar experiments where TNT was replaced by 2,4-dinitrotoluene, 2,6-dinitrotoluene, 2-nitrotoluene, or 4-nitrotoluene, growth, as estimated by turbidity increase, did not occur.

HPLC analysis showed that TNT was removed from the medium during growth (Fig. 1). TNT concentrations detected in initial samples were considerably lower than the levels of TNT added, presumably due to incomplete dissolution of TNT

in the methanol-water mixture used. Peaks corresponding to dinitrotoluene and nitrotoluene were not detected. Two peaks which may represent metabolites of TNT were detected. One of these (retention time, 7.3 min; λ_{max} , 224 and 380 nm) had an elution position and a UV-visible spectrum similar to those of products resulting from the action of cloned *E. cloacae* nitroreductase (3) on TNT (data not shown). It is likely that this peak represents one or more stable nitroreductase products, such as isomers of hydroxylaminodinitrotoluene, aminodinitrotoluene, or diaminonitrotoluene. Such products are commonly observed when bacteria are incubated with TNT (11). The second peak observed (broad absorbance peak; λ_{max} = 240 nm, falling to baseline at 400 nm) migrated at the solvent front in standard HPLC but was retarded by the column in ion-pair HPLC in the presence of the tetrabutylammonium counterion (retention time, 6.4 min), suggesting that this peak represents a negatively charged molecule. The nature of this molecule has yet to be determined. Several peaks with similar UV-visible absorption spectra were detected in aqueous TNT solutions which had been allowed to stand for prolonged periods (several weeks) or which had been treated with dilute NaOH (data not shown).

Following ethyl acetate extraction, samples were analyzed by gas chromatography. No peaks other than those of TNT and solvent were detected. It is noteworthy that peaks corresponding to 2,4-dinitrotoluene, 2,6-dinitrotoluene, and 2-nitrotoluene were not detected.

Reduction of TNT by PETN reductase. In PB2, degradation of nitrate esters is mediated by PETN reductase. To determine whether this enzyme might also play a role in TNT degradation, activity (NADPH oxidation) was measured with 0.05 mM TNT, 2,4-dinitrotoluene, 2,6-dinitrotoluene, 2-nitrotoluene, 4-nitrotoluene, or no substrate. The background rate of NADPH oxidation in the absence of substrate was 0.10 μmol of NADPH $\cdot \text{min}^{-1} \cdot \text{mg}$ of protein $^{-1}$. This rate was not enhanced in the presence of 0.05 mM 2,4-dinitrotoluene, 2,6-dinitrotoluene, 2-nitrotoluene, or 4-nitrotoluene. However, in the presence of 0.05 mM TNT, the observed rate of NADPH oxidation increased to 0.50 μmol of NADPH $\cdot \text{min}^{-1} \cdot \text{mg}$ of protein $^{-1}$, suggesting that TNT is able to oxidize the reduced form of the enzyme, presumably becoming reduced in the process. It was further observed that reaction mixtures containing PETN reductase, NADPH, and TNT developed an orange coloration, indicating the formation of a colored product from TNT. No such colored products were generated in the absence of enzyme, TNT, or NADPH or when nitroreductase replaced PETN reductase.

Nature of the products of TNT reduction. To investigate the nature of the orange product or products, a reaction mixture was set up containing 0.02 mg of PETN reductase/ml, 0.4 mM NADPH, and 0.5 mM TNT. Samples were analyzed by ion-pair HPLC. A similar experiment was performed with recombinant *E. cloacae* nitroreductase in place of PETN reductase (3).

During the reduction of TNT by PETN reductase, a UV-absorbing peak at a retention time of 7.7 min, corresponding to TNT, decreased. Another UV-absorbing peak at a retention time of 5.4 min appeared and increased in size. A peak with an identical retention time and absorbance spectrum was also observed when PETN reductase was replaced by nitroreductase. This peak is presumed to represent one or more nitroreductase products, such as isomers of hydroxylaminodinitrotoluene and/or aminodinitrotoluene, thus suggesting that PETN reductase has nitroreductase activity. With PETN reductase, six peaks with both UV and visible absorbances were detected, with retention times of 3.0 (peak A), 3.8 (peak B), 4.2 (peak C), 4.8 (peak D), 8.6 (peak E), and 11.6 min (peak F). These peaks were not observed with nitroreductase. Peak A

overlapped the peaks of NADPH and NADP $^{+}$, so that the shape of the spectrum below 400 nm could not be determined; however, the spectrum above 400 nm was identical to the spectrum of peak B in this region (visible λ_{max} , 470 nm; UV λ_{max} of peak B, 260 nm). The UV-visible spectra of peaks C and D appeared to be identical to one another (λ_{max} , 260 and 500 nm; asymmetrical visible peak with absorbance falling sharply above 500 nm, reaching baseline by 530 nm), as did the spectra of peaks E and F (λ_{max} , 250 and 480 nm; broad visible peak with shoulder at 550 nm; absorbance extending to above 600 nm).

When reaction mixtures were left for several hours, the observed peaks decreased in size, with no detectable peaks appearing to replace them. Visible color in the reaction mixtures also faded. This suggests that the colored products are further transformed to give nonaromatic (non-UV-absorbing) products. Alternatively, it is possible that highly soluble UV-absorbing products eluting at the solvent front, overlapping the peaks due to NADPH and NADP $^{+}$, may have been present.

When the samples were reanalyzed in the same mobile phase but with the tetrabutylammonium counterion omitted, all visible absorbance, presumably corresponding to peaks A, B, C, D, E, and F, eluted at the solvent front. The TNT and presumed nitroreductase product peaks were unaffected. This suggests that the visible peaks A to F represent negatively charged molecules.

The UV-visible spectra of peaks E and F were distinctive and were similar to the spectrum of the hydride-Meisenheimer complex of TNT (H $^{-}$ -TNT) reported in the literature (7, 14).

Following ethyl acetate extraction, samples from the enzymic incubation were analyzed by gas chromatography. No peaks other than TNT and solvent were detected.

Comparison with chemical reduction of TNT. To determine whether peaks E and F represented H $^{-}$ -TNT, authentic H $^{-}$ -TNT was prepared by chemical reduction of TNT with sodium borohydride (6, 7). To 1 ml of 10 mM TNT in acetonitrile was added 2.8 mg of solid sodium borohydride (NaBH $_4$). The reaction mixture instantly developed a deep brownish purple color, and the UV-visible spectrum, measured in 50% (vol/vol) acetonitrile and 50% (vol/vol) water, was identical to that reported for H $^{-}$ -TNT. However, after standing at room temperature for several hours, an orange color and a red precipitate developed in the reaction mixture. If water was added to the reaction mixture at an early stage, so that excess borohydride was consumed through reaction with water, or if a smaller amount of borohydride was initially provided, the purple color was stable over days and no orange color developed. This suggests that the orange color represents a slow further reduction of H $^{-}$ -TNT.

Chemical reaction mixtures were analyzed by ion-pair HPLC as described above. Initially, TNT disappeared and peaks identical to peaks E (large) and F (small) appeared. As the reaction proceeded and orange coloration developed, peaks with retention times and UV-visible spectra similar to those of peaks A, B, C, and D appeared. A large UV peak lacking visible absorbance also appeared at the solvent front. No peak corresponding to the nitroreductase product peak appeared.

Reduction of H $^{-}$ -TNT by PETN reductase. To confirm that the orange products were produced through further reduction of H $^{-}$ -TNT, H $^{-}$ -TNT was prepared chemically as described above from 2.3 mg of TNT, and the chemical reduction was quenched with aqueous buffer after 2 min. An enzymic reaction mixture was prepared containing 0.4 mM NADPH, 0.04 mg of PETN reductase/ml, and the chemical reduction mixture in 5 ml of buffer. The brown-purple color of the chemical re-

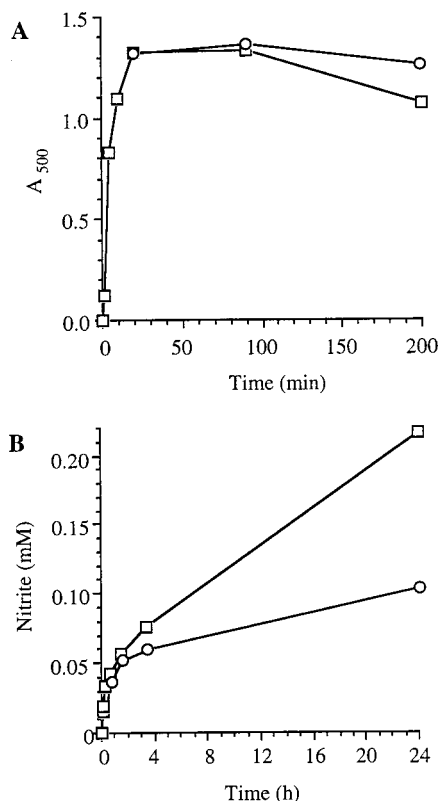


FIG. 2. Liberation of nitrite from TNT by purified PETN reductase. (A) Visible absorbance; (B) nitrite concentration. Squares, no progesterone; circles, 0.044 mM progesterone.

duction product, representing H^- -TNT, was rapidly replaced by an orange color identical to that seen in enzymic reduction of TNT by PETN reductase. The UV-visible absorbance spectrum of the reaction mixture was identical to that seen during enzymic reduction of TNT. Ion-pair HPLC analysis confirmed that products corresponding to peaks A, B, C, and D were formed. When nitroreductase replaced PETN reductase, the brown-purple color of H^- -TNT faded but was not replaced by the orange color seen with PETN reductase. No UV-absorbing product was detected by ion-pair HPLC, although, again, a product eluting near the solvent front might easily have been obscured by the peaks due to NADPH and $NADP^+$.

Liberation of nitrite from TNT by PETN reductase. It was noted that, during enzymic reduction of TNT by PETN reductase, nitrite was liberated. A reaction mixture was prepared containing 0.4 mM TNT, 2.0 mM NADPH, and 0.04 mg of PETN reductase/ml. After 25 min, the reaction mixture was split into halves. To one of these was added progesterone (0.044 mM final concentration), a potent inhibitor of PETN reductase activity (5).

The results are shown in Fig. 2. Over 200 min, 0.076 mM nitrite was released (0.19 mol of nitrite/mol of TNT), and after 24 h this had increased to 0.22 mM nitrite (0.54 mol of nitrite/mol of TNT). Addition of progesterone reduced the rate of loss of visible absorbance and the rate of nitrite formation, suggesting that further transformation of the orange products may be associated with enzyme activity.

In further experiments, NADPH recycling was obtained by the inclusion of 0.02 mg of *Thermoanaerobium brockii* $NADP^+$ -dependent alcohol dehydrogenase (Sigma)/ml and 1% (vol/vol) isopropanol. In such reaction mixtures which had been left

standing for several days, up to 1.0 mol of nitrite/mol of TNT was detected. It is not clear from these experiments whether this represents a limiting value.

Transformation of TNT by recombinant *E. coli* expressing PETN reductase. *E. coli* JM109/pONR1 was grown in a rich medium as previously described (5). Cells from a stationary-phase culture were harvested by centrifugation and resuspended in 1/10 of the original culture volume of 50 mM phosphate buffer, pH 7, containing 11 mM glucose and 0.5 mM TNT. A bright-orange coloration was immediately produced, and ion-pair HPLC confirmed the production of products represented by peaks A, B, C, and D. Nitrite was detected in supernatants by Griess assay; after 20 h of incubation, 0.4 mM nitrite was present (0.8 mol of nitrite/mol of TNT).

DISCUSSION

E. cloacae PB2 was found to grow in mineral medium with TNT as the sole nitrogen source. PETN reductase, responsible for the ability of *E. cloacae* PB2 to grow at the expense of nitrate esters as the sole nitrogen source, was found to reduce TNT. Two types of products were detected. Firstly, products resembling those produced by a classical nitroreductase (3) were seen. Secondly, TNT was reduced to H^- -TNT, which was further reduced to orange products.

Nitroreductase-like activity in PETN reductase is not surprising, since the aromatic nitro groups of TNT are extremely susceptible to reduction and can be reduced by a variety of oxidoreductases with redox-active prosthetic groups, such as flavins (3). The total reductive activity of PETN reductase with TNT was measured as 0.4 U/mg, far lower than the activity of *E. cloacae* nitroreductase (approximately 300 U/mg; data not shown). Reduction of TNT to H^- -TNT has previously been reported for whole cells of picrate-utilizing *Rhodococcus erythropolis* (15), 4-nitrotoluene-utilizing *Mycobacterium* sp. (14), and TNT-utilizing *Pseudomonas* sp. (4, 6), although the last has recently been contradicted (15). Similar reduction of the aromatic ring has been reported for di- and trinitrophenols (8, 9); however, to the best of our knowledge, this is the first report of production of H^- -TNT by a purified enzyme.

Curiously, our experiments with TNT reduction by PETN reductase and sodium borohydride showed two distinct ion-pair HPLC peaks with the distinctive UV-visible absorbance spectrum of H^- -TNT. Kaplan and Siedle (7) reported that the C-3 adduct dominates in reduction of TNT by boron hydrides. The smaller of our two peaks may represent the C-1 hydride adduct.

PETN reductase further reduces H^- -TNT to negatively charged orange products. The same products were observed in the reduction of TNT by sodium borohydride. In the reduction of TNT by borohydride, these products were produced slowly following very rapid reduction of TNT to H^- -TNT; by contrast, in enzymic reduction, H^- -TNT was seen only transiently, suggesting that reduction of H^- -TNT to the orange products was much more rapid than reduction of TNT to H^- -TNT.

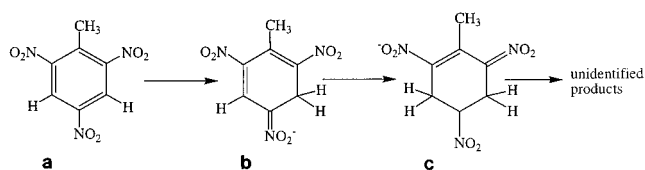


FIG. 3. Formation of H^- -TNT. (a) TNT; (b) C-3 H^- -TNT; (c) C-3,C-5 2 H^- -TNT.

Vorbeck et al. (15) reported reduction of TNT to H^- -TNT and of H^- -TNT to yellow products by whole cells of picrate-utilizing *R. erythropolis* and 4-nitrotoluene-utilizing *Mycobacterium* sp. The yellow products were identified as the protonated and dissociated forms of the C-3,C-5 dihydride-Meisenheimer complex of TNT ($2H^-$ -TNT). As in our experiments, the reduction of H^- -TNT to these products was more rapid than initial reduction of TNT to H^- -TNT. We identified four products of H^- -TNT reduction. These may represent the protonated and dissociated forms of the C-1,C-3 and C-3,C-5 dihydride adducts. The visible λ_{max} values of our products (470 nm for two products and 500 nm for two products) are rather higher than those reported by Vorbeck et al. (430 and 445 nm); this may be due to the higher ratio of acetonitrile to water in our ion-pair HPLC mobile phase, since our products showed reduced λ_{max} in solvents containing larger proportions of water (λ_{max} of the product mixture in aqueous buffer, 432 nm). Figure 3 illustrates the formation of H^- -TNT and $2H^-$ -TNT; however, further experiments are required to establish the identity of our products with certainty.

Interestingly, we detected the accumulation of nitrite in reduction mixtures simultaneously with the disappearance of the orange products. This suggests slow breakdown of the orange products with release of nitrite; alternatively nitrite may be eliminated from some other reaction product as yet undetected. The reduced rate of nitrite production in the presence of an inhibitor of PETN reductase activity suggests that this transformation may be enzyme catalyzed. Vorbeck et al. (15) failed to detect nitrite production from $2H^-$ -TNT by organisms capable of reducing TNT to $2H^-$ -TNT; this argues against spontaneous nitrite elimination of the type seen with hydride adducts of picric acid.

In experiments with TNT degradation by several organisms apparently capable of utilizing TNT as the sole nitrogen source, Vorbeck et al. (15) found that the ability to utilize TNT diminished with successive subculturing, suggesting that sustained use of TNT as the sole nitrogen source might not occur in these strains. In view of these results, further experiments are required to establish beyond doubt that *E. cloacae* PB2 can grow indefinitely with TNT as the sole nitrogen source; however, in view of the production of nitrite from TNT by PETN reductase and by recombinant *E. coli* overexpressing PETN reductase, extraction of nitrogen from TNT by PB2 is plausible. Further experiments to test the role of PETN reductase in TNT utilization will involve the inactivation of the PETN reductase gene in PB2 and its expression in other nitrite-utilizing bacteria.

To the best of our knowledge, this is the first report of either the reduction of the aromatic ring of TNT or the liberation of nitrogen from TNT by a purified enzyme. Since the final reac-

tion products of TNT reduction by PETN reductase contain less nitrogen than TNT and appear to be water soluble and nonaromatic, they are likely to be less toxic and less recalcitrant than TNT or nitroreductase products of TNT. Therefore, *E. cloacae* PB2 and recombinant organisms expressing PETN reductase may be useful in the bioremediation of TNT-contaminated soil and water.

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